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Influence of temperature preferences of two *Paecilomyces fumosoroseus* lineages on their co-infection pattern

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Abstract

In order to clarify the epidemiological potential of entomopathogenic fungi for insect pest control, the role of the temperature as one environmental constraint was investigated on the pattern of co-infection of *Galleria mellonella* by two distinct lineages of a hyphomycete, *Paecilomyces fumosoroseus*. The distribution of conidial populations collected on cadavers of hosts co-infected under 20 regimes, ranging from 13 to 35 °C, was examined. The apparent temperature tolerance of both fungal isolates was related to their in vitro colony growth and their in vivo sporulation ability. The conidial populations were characterized by molecular markers based on restriction fragment length polymorphisms of the internal transcribed spacers (ITS-RFLP) and random amplified polymorphic DNA (RAPD) contrasting profiles in combination with the conidial size. This study allowed a different temperature profile was identified for each isolate. Under most temperature regimes, only one lineage prevailed upon the infected insect; whereas both lineages coexisted at 20–25 and 25–25 °C. When one haplotype dominated, the displacement of the other one depended on its temperature tolerance. These results suggest that more consideration should be given to population-genetics analyses for evaluating the adaptability of microbial control agents to targeted environments.
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1. Introduction

The entomopathogenic hyphomycete, *Paecilomyces fumosoroseus*, has recently become commercially available as a mycoinsecticide primarily against the greenhouse whitefly, *Trialeurodes vaporariorum* (Homoptera: Aleyrodidae) (Bolckmans et al., 1995; Osborne and Landa, 1992). More recently, it has also been recognized as a promising candidate for biocontrol of the silverleaf whitefly, *Bemisia tabaci argentifolii*, which is a worldwide major insect pest in field and greenhouse crops (Lacey et al., 1996). Critical to commercial viability of a mycoinsecticide on the plant protection market is the choice of a high quality product that causes high mortality in the targeted pest populations under the environmental conditions prevailing in the regions where it is used (Milner et al., 2003). Pathogenic activity has long been viewed as the predominant feature for selecting fungal strains for biological control projects, and it is assumed that certain fungal genotypes evolve with an insect host (Couteaudier et al., 1998). More consideration is now being given to environmental preferences of the pathogens. Bidochka et al. showed that genetic groups of *Metarhizium anisopliae* (Bidochka, 2001) and of *Beauveria bassiana* (Bidochka et al., 2002) were associated with habitat and temperature preferences and not with insect hosts. These findings provide evidence that

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the adaptation of an entomopathogen to an environment has strong implications upon its efficiency as a biocontrol agent. Recently, the role of climatic pressures in fungal epizootics has not only been recognized as crucial for selecting environmentally well-adapted pathogens, but also for evaluating the risks of competition between released and native fungal populations (Fargues et al., 2002; Milner et al., 2003).

Paecilomyces fumosoroseus is distributed world-wide in a large range of climates from tropical, subtropical to temperate, all differing significantly in their temperature, and humidity conditions (Lacey et al., 1996). Although both climatic conditions are critical to the selection of the most adapted strains under particular target environments, temperature tolerance is the trait which has been the most considered (Fargues et al., 1992; Roberts and Campbell, 1977). Vidal et al. (1997a) have addressed the variability of temperature tolerance among *P. fumosoroseus* isolates of various insect host origins collected by Lacey et al. (1996) in different areas in North America and Eurasia. In that study, Vidal et al. (1997a) found a close relationship between the temperature tolerance of the isolates and the environment from which they originated from. Recently, the genetic relatedness of these *P. fumosoroseus* isolates was assessed, based on both RFLP-ITS analysis and ITS sequencing data. This genetic analysis showed a clustering of the isolates into three distinct lineages, one being strictly associated with the host *Bemisia* spp. (Fargues et al., 2002).

In the present study, two isolates, belonging to different lineages identified from the previous analysis reported by Fargues et al. (2002) and differing in both their conidial size (Fargues, unpublished) and temperature tolerance (Vidal et al., 1997a) are compared. The aim of this study was to further evaluate the role of temperature as one environmental constraint on the infection dynamics of entomopathogenic fungi. The pattern of co-infection of *Galleria mellonella* by these two distinct lineages of the hyphomycete *P. fumosoroseus* is examined under various cycling temperature regimes, ranging from 13 to 35 °C. The temperature tolerance of the isolates is estimated both by their vegetative growth and their in vivo sporulation ability. Populations were characterized by their conidial sizes and by molecular markers, viz. restriction fragment length polymorphisms of the internal transcribed spacers (ITS-RFLP) and random amplified polymorphic DNA (RAPD) profiles.

2. Materials and methods

2.1. Fungal isolates and mycological techniques

2.1.1. Fungal isolates

Two *P. fumosoroseus* strains were selected from both I.N.R.A. and A.R.S.E.F. collections of entomopathogenic fungal cultures (Institut National de la Recherche Agronomique Fungal Collection, La Minière, France and Agricultural Research Service Entomopathogenic Fungal Cultures, USDA, Ithaca, NY, respectively). The strain *Pfr11* (=INRA 35) was isolated from a larval cadaver of *Mamestra brassicae* (Lepidoptera: Noctuidae) collected in France in a temperate area (Ile-de-France) (Fargues et al., 2002), whereas the strain *Pfr46* (=ARSEF 4501) was collected from *Bemisia tabaci* (Homoptera: Aleyrodidae) in Pakistan in a dry tropical area (Multan) (Lacey et al., 1996).

2.1.2. Conidial size studies

The length of the conidia was measured by using a micrometer under a light microscope at 900× magnification. Per each single-spore colony, the length of thirty conidia was measured.

2.1.3. Radial growth studies

Surface radial growth (Horsfall, 1956) was determined according to the method described by Fargues et al. (1992) and Vidal et al. (1997a). Three Petri dishes were incubated for 12 days in the dark, under saturated humidity for each of the 20 tested temperature conditions. Temperature regimes consisted of six constant conditions, 13, 20, 25, 28, 32, and 35 °C and fifteen 12:12 h cycles, ranging from 13–20 to 32–35 °C (13–20, 13–25, 13–28, 13–32, 13–35, 20–25, 20–28, 20–32, 20–35, 25–28, 25–32, 25–35, 28–32, 28–35, and 32–35 °C) according to previous results (Vidal et al., 1997a). Surface radial growth was recorded daily by using two cardinal diameters, through two orthogonal axes previously drawn on the bottom of each dish. Original data consisted of means of these two daily measurements. Growth assays were carried out twice.

2.2. Molecular characterization

2.2.1. DNA extraction

Genomic DNA was isolated from mycelial mat of 2-day old monoconidial cultures as previously described by Fargues et al. (2002).

2.2.2. PCR-RFLP and sequencing of the ITS region

A rDNA fragment including ITS1 and ITS2 and the 5.8S rDNA gene was amplified by PCR using the complementary primers ITS1F and ITS4A (Larena et al., 1999). Amplifications were run in a MJ Research PTC-100 thermocycler according to the conditions described in Fargues et al. (2002). The fragment corresponding to the rDNA-ITS region was digested separately by *Alu*I (10 U μl^{-1}), *Hae*III (10 U μl^{-1}), *Hin*6I (10 U μl^{-1}), *Hpa*II (8 U μl^{-1}), *Nde*II (10 U μl^{-1}), and *Sma*I (10 U μl^{-1}) as previously reported for several *P. fumosoroseus* isolates (Fargues et al., 2002). Both strands of each PCR product were sequenced by Genome Express (Grenoble, France)

in an Applied Biosystems 3753 DNA sequencer. The sequences of the isolates examined in this study were aligned using ClustalX program, version 1.81 (Thompson et al., 1997).

2.2.3. RAPD-PCR analysis

Random amplification of polymorphic DNA was performed in a Hybaid thermocycler (Hybaid, PCR Express, USA). Conditions were 5 min at 95 °C, 30 cycles of 1 min at 92 °C, 1 min at 36 °C, 1:30 min at 72 °C, with a final additional cycle of 15 min at 72 °C. Reactions were carried out in a total volume of 12.5 μl containing Qiagen PCR buffer with 1.5 mM $MgCl_2$, 400 μM of each dNTP, 10 pmol of each primer, 1.25 U *Taq* DNA polymerase (Qiagen, France), and 10 ng DNA template. Primers and sequences for RAPD were: OPA-07 (5′-GAAACG GGTG), OPA-09 (5′-GGGTAACGCC), OPA-13 (5′-C AGCACCCAC), OPA-14 (5′-TCTGTGCTGG) (Operon, USA). These primers were chosen because they generated the most polymorphic patterns for the two isolates tested (Fargues et al., 2002). Products were separated on a 1.4% agarose gel in 0.5× TAE (20 mM Tris–acetate, 0.5 mM EDTA, and pH 8.0) and visualized by staining with ethidium bromide and photographed over a transilluminator.

2.3. Insect culture

Larvae of *G. mellonella* (Lepidoptera: Pyralidae) were obtained from a strain maintained at the INRA Comparative Pathology Research Laboratory, St Christol (France). They were reared at 28 °C and fed with a mixture of wax and pollen. Cohorts of sixth-instar larvae of weights ranging from 140 to 180 mg were randomly selected from the stock of wax moth larvae.

2.4. Experimental procedures

2.4.1. Assays of co-infection under twenty temperature regimes

2.4.1.1. Rationale of the experimental procedures. *Galleria mellonella* larvae were selected for co-inoculation bioassays for two reasons. First, *G. mellonella* larvae do not depend on the type of substrates used unlike plant-feeding *Bemisia* larvae, which adhere to the under-leaf surfaces. Second, their size is suitable for the inoculation of calibrated conidial suspensions by injection. This mode of inoculation was preferably used for eliminating the first host barrier to fungal infection process, in order to avoid the prevalence of one strain (Wang et al., 2002). In spite of their susceptibility towards entomopathogenic hyphomycetes (Zimmermann, 1986), *G. mellonella* larvae were significantly less susceptible to *Pfr11* than to *Pfr46*, when inoculated topically (Fargues, unpublished data). Hence, the effect of the host specificity which was not the issue of this study, is minimized by this mode of inoculation. Infection required a relatively high conidia doses (10^4 per larva) to kill larvae, due to a residual role of the host pressure as previously demonstrated with other insect–pathogen combinations (Fargues, 1976; Ignoffo et al., 1982; Vey et al., 1982). There is some circumstantial evidence that the temperature has a strong effect on the fungal sporulation intensity on cadavers of various insect species (Fargues and Luz, 1998; Luz and Fargues, 1998). Preliminary assays have shown an irreversible effect of the temperature on the fungal development during the infection phase in the living *Galleria* larvae under conditions allowing the sporulation of the two isolates, i.e., over a range of 15–28 °C (Fargues, unpublished data). Hence, the exposure to the different temperature regimes was not extended to the non parasitic phase of the fungus inside the cadavers. Because of putative interactions with saprophytic fungi under temperature conditions unfavorable to one of the two isolates (13 °C for *Pfr46* and 35 °C for *Pfr11*), host-produced inocula were re-isolated for providing single-spore colonies. The first series of assays were then carried out on the in vitro produced conidia. This protocol was developed based on results obtained from preliminary assays that showed no significant differences in the conidial distribution of the two lineages between direct observation of host-produced conidia and secondary conidia collected from a single-spore colony. The re-isolation did not result in any directed genetic change as confirmed by the results obtained from both the RFLP-ITS and RAPD analyses.

2.4.1.2. Experimental design. In the first series of co-infection assays, four batches of 10 larvae were tested for each temperature-inoculum combination. All experiments were repeated twice. Assays consisted of exposing co-inoculated *G. mellonella* larvae, and reference batches of larvae inoculated with only one *P. fumosoroseus* isolate or with sterile water, to different temperature regimes. These regimes consisted of six constant conditions, ranging from 13 to 35 °C, and fifteen 12:12 h cycles, ranging from 13–20 to 32–35 °C, as listed above for growth assays.

2.4.1.3. Bioassay procedure. Conidia of *P. fumosoroseus* were obtained from 2-week-old sporulating cultures grown at 25 + 1 °C on agar slants of semi-synthetic medium (KH_2PO_4, 0.36 g; $Na_2HPO_4 \cdot 12H_2O$, 1.42 g; $MgSO_4 \cdot 7H_2O$, 0.6 g; KCl, 1.0 g, NH_4NO_3, 0.7 g; glucose, 10 g; yeast extract, 5.0 g chloramphenicol, 0.5 g agar, 18 g; and 1000 ml H_2O). Conidia were collected directly from the surface of these cultures by scraping. Inocula were suspended in 10 ml sterile distilled water by shaking (700 oscillations/min) in 45-ml flasks containing 5–6 dozen glass beads (3 mm in diameter) for 5 min. Conidial suspensions for bioassays were then adjusted to 2×10^5 conidia/ml based on hemocytometer counts.

Bioassays were carried out by using for each tested temperature condition four treatments of four replications each of 10 sixth-instar larvae of *G. mellonella* larvae. The first treatment consisted of larvae injected with sterile distilled water as controls, whereas the three other treatments consisted of larvae injected with suspension of conidia of *Pfr11* or of *Pfr46*, individually, or with a mixture (0.5:0.5) of both inocula. Inoculation consisted in injecting 20-μl aqueous suspensions of conidia (10^4 conidia/larva) of each of the two isolates (10^4 conidia of *Pfr11* or of *Pfr46*) or of a mixture of both inocula (5×10^3 conidia of *Pfr11* and 5×10^3 of *Pfr46*) through the second proleg into the hemocoel of each hand-held immobilized larva. Treated larvae of each cohort were then individually placed in compartmentalized plastic boxes ($130 \times 90 \times 25$ mm with twelve compartments of $32 \times 39 \times 24$ mm), previously garnished with individual wax pieces of ca. 400 mg. Cohorts were held in temperature-regulated incubators and larval mortality was recorded daily for 12 days. All fungus-inoculated insects died over a 10-day period.

2.4.1.4. Incubation of fungus-killed insects. After death, fungus-killed insects were externally disinfected by bathing in 70% ethanol solution for 15 s, and then dried on filter paper. At day 10, cadavers were then placed in plastic boxes ($27 \times 36 \times 18$ cm) and transferred to controlled-temperature chambers for sporulation in the dark at 15 ± 1 °C. After ca. 7 days of incubation, two sporulated cadavers were randomly sampled from each lot of 10 fungus-killed larvae. Conidia were harvested from each cadaver by shaking (700 oscillations/min) for 1 min in 10-ml sterile distilled water in 45-ml flasks, without glass beads to avoid bursting of the cadavers. After filtering through a 90-μm screen, 0.5 ml aliquots were plated on nutrient agar using a spiral plating method (Gilchrist et al., 1973) and incubated at 25 °C. After 4-day incubation, 10 single-spore colonies were isolated for seeding 10 single-spore cultures. Each of these 10 single-spore cultures was screened for conidial size by observation with a micrometer under 900× magnification.

Size-based information on conidial population structure on cadavers of co-infected larvae exposed, respectively at 20–25 and 25–25 °C, was verified by using PCR-RFLP of rDNA-ITS of two randomized single-spore cultures of both conidial size types per sampled host cadaver per temperature condition. Comparative analysis of the nucleotide sequences of the rDNA-ITS was established for a couple of both short- and long-conidia single spore culture from two randomly selected cadaver of co-infected larvae exposed at 25–25 °C. RAPD analysis was also done on the single-spore cultures selected for rDNA-ITS sequencing and on one single spore culture of each reference isolates not passed through *G. mellonella*.

2.4.2. Assays of co-infection under favorable temperature regimes

In the second series of co-infection assays, experiments were replicated four times, and one batch of 30 larvae was used in each temperature-inoculum combination for each replication. These co-infection assays were carried out for analyzing the distribution of the two types in conidial populations collected on cadavers of larvae co-infected at four temperature regimes, 20–20, 20–25, 25–25, and 25–28 °C, respectively. The protocol was similar to that of the first series except the number of larvae per batch, which was increased to 30 because of statistical requirements for frequency analysis. Moreover, host-produced conidia were directly examined by measuring the length of 100 conidia in suspensions provided by each of the 30 cadavers.

2.5. Statistical analysis

Radial growth data were subjected to regression analysis (SPSS, 1997). Because radial measurements (from the 3rd to the 12th days) fit a linear model ($y = vt + b$), growth rates (velocity in mm day^{-1}) were used as the main parameter to evaluate the influence of temperature regime on fungal growth (Fargues et al., 1992; Vidal et al., 1997a). The growth rate per isolate and per temperature regime was then expressed as the mean growth rate of five cultures selected for the regularity of the contour of the fungal colony among the six transplanted Petri dishes. ANOVAs were done on these absolute growth rates (in mm day^{-1}) for each fungal isolate under various temperature regimes and then, means were compared in using the Student–Newman–Keuls (SNK) multiple range test. Because of intrinsic differences in growth capacity on nutrient agar between the two tested fungal isolates, relative growth rates (in %) calculated from the maximum growth rate for each isolate, were also used to compare isolates.

Coinfection. In the first series of co-infection assays, the length of conidia was analyzed using the Sigmastat software 5 SPSS, 1997 from averages of 30 conidia per sample. Each set of averaged data consisted in 10 averaged conidial lengths of the 10 single-spore cultures from two randomly selected cadavers per experimental condition, per replicate, i.e., 40 sets of averaged spore-length data.

In the second series, the length of 100 conidia per cadaver was examined for estimating the proportion of each conidial-size-based haplotypes. The χ^2 analysis of contingency tables was used for comparing the number of cadavers belonging to four categories related to the distribution of the short-conidia haplotype (ITS-H11) in their conidial population (0–30, 31–60, 61–90, and >91%) issued from larvae co-infected with both *Pfr11* and *Pfr46* isolates.

3. Results

3.1. Phenotypic traits of both Pfr11 and Pfr46 isolates

3.1.1. Conidial size

The length of the aerial conidia of *Pfr11* was 3.65 ± 0.01 μm and that of *Pfr46* 5.23 ± 0.08 μm, when these fungal isolates were grown at 25 °C on semi-synthetic nutrient agar. Aerial conidia differed significantly in length ($F_{1,\ 78} = 6{,}619.81$, $P < 0.001$) and their size distributions showed less than 5% of concurrence (Fig. 1).

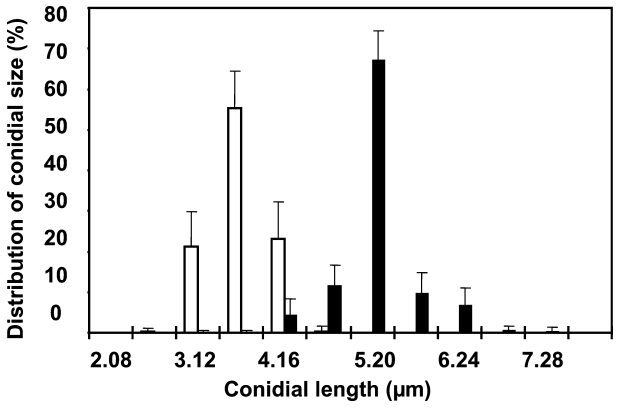

Fig. 1. Distribution of the conidial size of the reference isolates of *P. fumoroseus*, *Pfr11* (white bars) and *Pfr46* (black bars). Error bars represent the standard deviation ($P = 0.05$).

3.1.2. Thermal tolerance

The radial growth of both *P. fumosoroseus* isolates fits the linear model from the 3rd to the 12th day after inoculation of the plates under fluctuating temperature regimes, as well as under constant conditions. The coefficients of correlation (r^2) were higher than 0.920 under most temperature regimes for both fungal isolates, excepted for *Pfr11* at 13–32 °C (r^2: 0.623–0.795), 13–35 °C (r^2: 0.485–0.638), 28–35 °C (r^2: 0.679–0.756), and 32–32 °C (r^2: 0.688–0.911). Mycelial growth rates of *Pfr11* varied from 0 at 32–35 °C to 4.11 ± 0.09 mm day^{-1} at 20–25 °C, and those of *Pfr46* from 0.05 ± 0.02 at 32–35 °C to 3.91 ± 0.02 mm day^{-1} at 25–28 °C (Table 1). At lower and intermediate temperatures ranging from 13–13 to 20–25 °C, the relative growth rates of *Pfr11* (40–100%) were higher than those of *Pfr46* (23.5–79.3%). In contrast, at higher temperatures (from 25–25 to 32–35 °C) and under temperature regimes combining a low temperature period (13 °C) with a high temperature period (from 28 to 35 °C), both absolute and relative growth rates of *Pfr46* were significantly higher than those of *Pfr11*, except at 13–28 °C (Table 1). Temperature regimes including a high temperature of 35 °C dramatically decreased the growth rate of the European isolate, *Pfr11* (maximum at 25–35 °C: $v = 0.70 \pm 0.04$ mm day^{-1}, i.e., 17%, instead of $v = 1.48 \pm 0.10$ mm day^{-1}, i.e., 37.9%, for *Pfr46*).

Table 1
Effect of daily temperature regimes (*T*1–*T*2) and constant reference temperatures on radial growth rates (mm day^{-1}) of two *P. fumosoroseus* isolates, *Pfr11* and *Pfr46*, belonging to two distinct rDNA-ITS haplotypes

Isolate	Radial growth rates (mm day^{-1})*						
	T1 (12 h) (°C)	T2 (12 h)					
		13 °C	20 °C	25 °C	28 °C	32 °C	35 °C
Pfr11	13	1.65 ± 0.07 n (40%)	2.39 ± 0.03 j (58.2%)	3.07 ± 0.05 g (74.7%)	2.43 ± 0.02 ij (59.1%)	0.70 ± 0.06 r (17.0%)	0.03 ± 0.02 v (0.7%)
Pfr46	13	0.92 ± 0.01 q (23.5%)	1.69 ± 0.04 m (43.2%)	2.52 ± 0.02 i (64.5%)	2.37 ± 0.04 j (60.6%)	1.63 ± 0.03 n (41.7%)	0.34 ± 0.12 t (8.7%)
Pfr11	20		3.79 ± 0.02 c (92.2%)	4.11 ± 0.09 a (100%)	3.30 ± 0.05 e (80.3%)	1.12 ± 0.02 p (17.3%)	0.40 ± 0.04 t (9.7%)
Pfr46	20		2.82 ± 0.10 h (72.1%)	3.10 ± 0.09 g (79.3%)	3.52 ± 0.08 d (90.3%)	2.51 ± 0.02 i (64.2%)	0.56 ± 0.04 s (14.3%)
Pfr11	25			3.74 ± 0.06 c (91.0%)	3.14 ± 0.08 f (76.4%)	0.62 ± 0.02 rs (15.1%)	0.70 ± 0.04 r (17.0%)
Pfr46	25			3.84 ± 0.02 b (98.2%)	3.91 ± 0.02 b (100%)	2.83 ± 0.10 h (72.4%)	1.48 ± 0.10 o (37.9%)
Pfr11	28				2.02 ± 0.04 l (49.1%)	0.36 ± 0.05 t (8.8%)	0.02 ± 0.01 v (0.5%)
Pfr46	28				3.90 ± 0.02 b (99.7%)	2.21 ± 0.06 k (56.5%)	0.64 ± 0.03 rs (16.4%)
Pfr11	32					0.18 ± 0.15 u (4.4%)	0 (0%)
Pfr46	32					1.47 ± 0.04 o (37.6%)	0.05 ± 0.02 v (1.3%)

* Linear mycelial growth rates (velocity in mm per day from $y = vt + b$) established from each replicate. Rate data are means of five replicates (±SD). One–way ANOVA for temperature effect: $F = 1882.67$; $df = 33, 136$; $P < 0.001$. Means followed by different letters are significantly different ($P < 0.05$) according to the SNK multiple range test. Relative growth rates are mentioned in brackets.

3.2. Effects of temperature on distribution of fungal populations on host cadavers

3.2.1. Host mortality response to infection

With the inoculum dose used, all injected larvae died and the lethal time of the larvae in response to infection by the two tested isolates did not differ at medium temperature regimes. In both cases, the first deaths occurred on day 3 under favorable temperature conditions as compared to 8–9 days under less favorable temperatures. In spite of these differences of incubation time, all larvae in each group died in less than 48 h. Under unfavorable temperature regimes, including a 35 °C phase, 100% mortality was observed in larvae injected with *Pfr46* and with the mixture of both fungal inocula, except at 13–35 and 35–35 °C. Moreover, fungal sporulation occurred in cadavers of infected larvae exposed to 20–35 °C, but there was no sporulation on cadavers issued from larvae exposed to 25–35, 28–35, and 32–35 °C. In contrast, under temperature regimes including a 35 °C phase, larvae injected with *Pfr11* and control larvae survived.

3.2.2. Phenotypic analysis of conidial populations

The size of *Pfr11* conidia produced on *G. mellonella* cadavers varied from 3.41 to 3.81 μm and that of *Pfr46* from 4.45 to 5.23 μm. Under temperature regimes below 13–28 °C and above 28–32 °C, both isolates exhibited shorter conidia than under favorable temperature conditions (25–25 and 25–28 °C), but the difference in the conidial size between the two isolates was always significant (Fig. 2). The isolate *Pfr11* was able

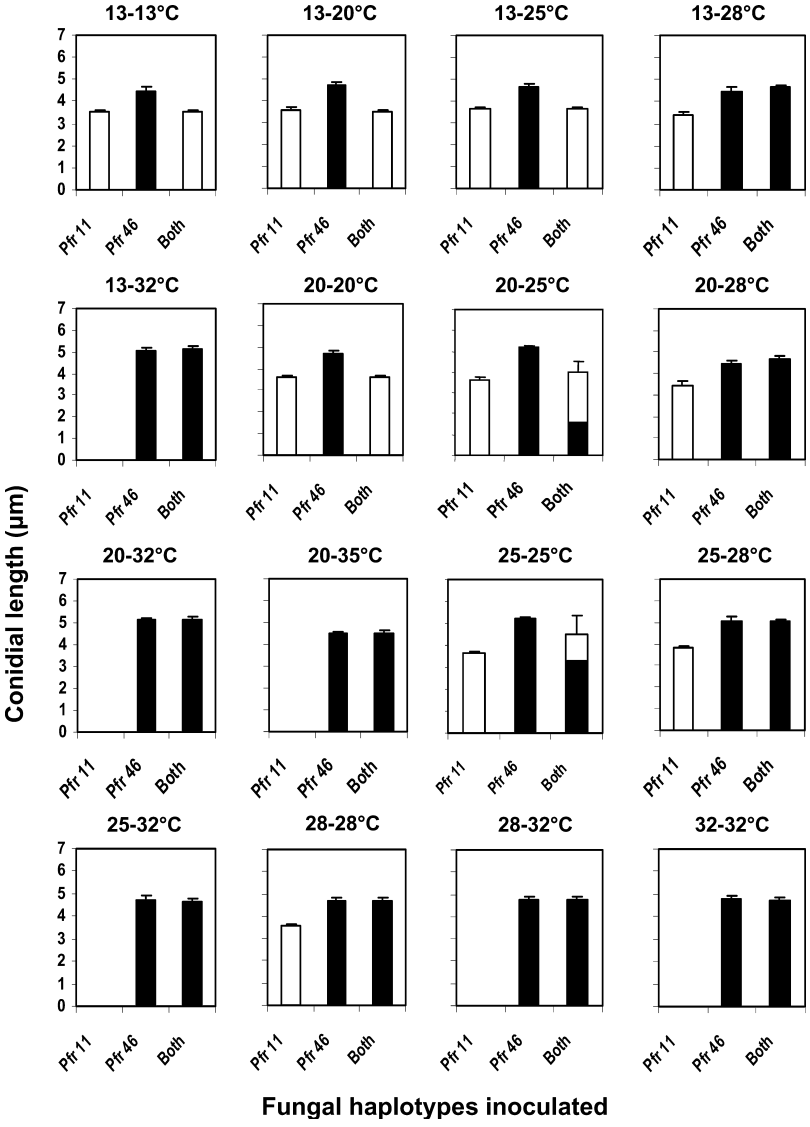

Fig. 2. Effect of temperature regimes on the distribution of the size of conidia collected on cadavers from *G. mellonella* larvae infected with either *Pfr11* or *Pfr46*, or co-infected with both fungal isolates. Length of conidia with standard deviation ($P = 0.05$). Relative distribution of short-type conidia (white bars) and long-type conidia (black bars).

to sporulate on cadavers of individuals exposed to temperature regimes ranging from 13 to 28 °C, but it failed under regimes including 32 °C. In contrast, *Pfr46* sporulated under all regimes combining temperatures ranging from 13 to 32 °C, and even at 20–35 °C.

The size of conidia harvested from cadavers of co-inoculated larvae was highly variable depending on the temperature regime (Fig. 2). At lower temperatures (13–13 to 25 °C, and 20–20 °C), cadavers exhibited short-type conidia, which did not significantly differ from that of *Pfr11*, whereas at higher temperatures (28–20 to 28 °C, 32–13 to 32 °C, and 35–20 °C), cadavers provided long-type conidia similar to that of *Pfr46*. When exposed to 20–25 and 25–25 °C regimes, the length data means of conidia harvested from co-inoculated cadavers were 4.01 ± 0.52 and 4.50 ± 0.83 μm, respectively. They differed significantly from that of both *Pfr11* and *Pfr46* isolates (Fig. 2). The distribution of the size of the conidia harvested from four cadavers for each of these two temperature regimes showed that the apparently intermediate size resulted in the co-existence of conidia of both short- and long- types (Fig. 3).

The comparison of the number of co-inoculated cadavers belonging to four categories relating to the proportion of the short-type conidia lineage (H11) in their conidial population (0–30, 31–60, 61–90, and >91%) showed that temperature acts as a strong selection pressure ($\chi^2 = 680.008$; $df = 9$; $P < 0.001$) (Table 2). Each cell in the Table 2 listed the cumulative number of individuals, recorded in four replicates of 30 larvae, for that combination of category and group.

At 20–20 °C, all cadavers issued from co-infected larvae presented more than 91% of short-type conidia. In contrast, at 25–28 °C, there were less than 30% because of the abundance of long-type conidia. Analysis of conidial populations harvested from cadavers issued from co-infected larvae exposed to 20–25 and 25-25 °C ($\chi^2 = 48.263$; $df = 3$; $P < 0.001$) revealed that temperature controlled the proportion of each haplotype population, when adjusted for the temperature requirements of the co-infecting fungal isolates. The proportion of cadavers with more than 91% of short-type conidia dropped from 64% at 20–25 °C to 29% at 25–25 °C.

3.2.3. Genetic analysis of conidial populations

ITS-restriction patterns generated by six restriction endonucleases, *Alu*I, *Hae*III, *Hin*6I, *Hpa*II, *Nde*II, and *Sma*I, and of single-spore cultures (two short-conidia and two long-conidia isolates) started from conidia produced on cadavers of co-inoculated larvae exposed to 20–25 and 25–25 °C regimes showed no difference with restriction patterns obtained from the reference isolates (*Pfr11* and *Pfr46*, respectively) used to co-infect the larvae (Fig. 4). Further analysis of 20 single-spore isolates of both size-type conidia collected on co-inoculated insects confirmed this result (data not shown).

Comparison of rDNA-ITS sequences of the two original isolates (GenBank Accession Nos. AF461746 for

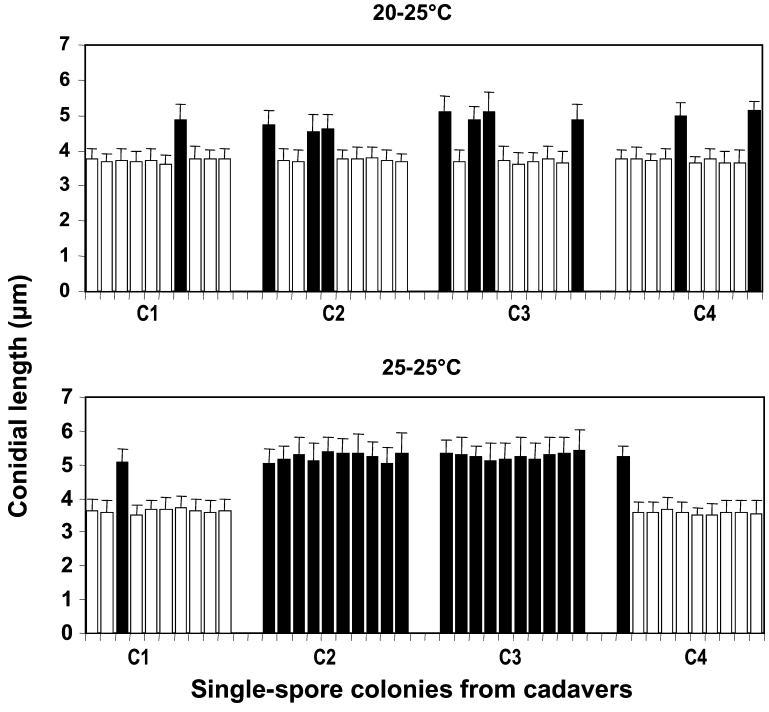

Fig. 3. Effect of temperature regimes (20–25 and 25-25 °C) on the distribution of the size of conidia collected on four larval cadavers (C1–C4) of *G. mellonella* larvae co-infected with *Pfr11* and *Pfr46*. Distribution of conidial sizes (smaller as white bars, and larger as black bars) from 10 single spore colonies per cadaver. Error bars represent the standard deviation ($P = 0.05$).

Table 2
Influence of the temperature regimes on the distribution of two lineages, ITS-H11 (conidial length = 3.66 ± 0.14 μm) and ITS-H46 (conidial length = 5.04 ± 0.24 μm) of *P. fumosoroseus* co-infecting *G. mellonella* larvae: distribution, in four classes, of cadavers[a] relative to the frequency of the haplotype ITS-H11 (short-type conidia) in the conidial population[b] harvested from each cadaver

Temperature regimes (°C)	Proportion of the ITS-H11 haplotype per cadaver			
	0–30 (%)	31–60 (%)	61–90 (%)	>91 (%)
Proportion of cadavers relative to the distribution of the haplotype ITS-H11[c,d,e]				
20–20	0 ± 0 (0)	0 ± 0 (0)	0 ± 0 (0)	100 ± 0 (120)
20–25	0 ± 0 (0)	2.5 ± 2.5 (3)	33.3 ± 19.7 (40)	64.2 ± 21.6 (77)
25–25	1.7 ± 1.7 (2)	23.4 ± 17.8 (28)	50.7 ± 16.7 (61)	24.2 ± 14.2 (29)
25–28	100 ± 0 (120)	0 ± 0 (0)	0 ± 0 (0)	0 ± 0 (0)

[a] Analysis of the conidial populations of four replicates of 30 cadavers per temperature regime.
[b] Conidial population unit = samples of 100 conidia per cadavrer.
[c] Averaged data ($x \pm$ SEM) expressed in percentages of cadavers in four replicates of 30 larvae per temperature regime are related to relative frequencies of the ITS-H11 haplotype based on screening of samples of 100 conidia per cadaver.
[d] In brackets, cumulative number of cadavers of four replicates per class of distribution of the ITS-H11 haplotype in conidial populations.
[e] Comparison of the cumulative number of cadavers per class of haplotype distribution in conidial populations under four temperature regimes: $\chi^2 = 680.008$; $df = 9$; $P < 0.001$. Comparison of data from assays at 20–25 and 25–25 °C, $\chi^2 = 48.263$; $df = 3$; $P < 0.001$.

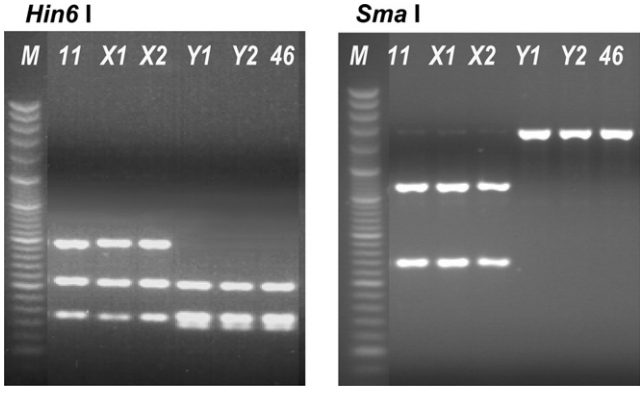

Fig. 4. Patterns within the *P. fumoroseus* ITS-rDNA region after digestion with *Hin*6I and *Sma*I, restriction endonucleases: comparison of the reference isolates, *Pfr11* and *Pfr46*, and four single spore isolates, *X1*, *X2*, *Y1*, and *Y2* (*X*, small-size conidia; *Y*, large-size conidia) collected on two cadavers (1 and 2) of co-infected *G. mellonella* larvae. The Sigma PCR Low Ladder Market Set (20 + 100 bp Ladder) was used as molecular weight marker (*M*).

Pfr11, and AF461744 for *Pfr46*) with the two short-conidia isolates (*X1* and *X2*), and the two long-conidia isolates (*Y1* and *Y2*) confirmed the similarity of the isolates within each of these two conidial size-based groups with conidia issued from bioassays carried out at 25–25 °C (Fig. 5) and at 20–25 °C.

RAPD fragments patterns from those reference isolates not passed through *G. mellonella*, and from the single spore colonies originated from cadavers of single-infected larvae (*Pfr11* and *Pfr46*) and co-infected larvae (*X1* for short-type conidia, and *Y1* for long-type conidia) at 25–25 °C (Fig. 6) and at 20–25 °C were generated with four primers, OPA-07, OPA-09, OPA-13, and OPA-14. Fig. 6 shows that RAPD profiles of short-type conidia isolates, reference *Pfr11*, host-passed *Pfr11*, and *X1*, were distinct from those of long-type conidia isolates, reference *Pfr46*, host-passed *Pfr46*, and *Y1*, and hence they are diagnostic for each group. In contrast, there were no differences in the RAPD patterns for any of the primers tested between isolates of the same morphological group.

4. Discussion

The entomopathogenic hyphomycetes are known to be mesophilic since most of the isolates tested so far grow over a range of 8 to 30–32 °C, with thermal optima ranging from 20 to 30 °C, and limits at <5 and >35 °C (Ferron et al., 1991; Fargues et al., 1992; Masuda and Kikuchi, 1992; Mietkiewski et al., 1994; Vidal et al., 1997a). In the present study, we have shown that thermal tolerance of the two isolates *Pfr11* and *Pfr46* were related to their history, including the geo-climatic origin. In the *Pfr11*-originated area in France, maximum average temperatures were ca. 25 °C in July, whereas temperature reached to 27–42 °C in the Pakistani area, where *Pfr46* originated (L. Lacey, personal communication). In contrast, in winter, median temperatures dropped to 0–5 °C and to 5–20 °C in these areas, respectively. Similar relationships between lower and upper temperature thresholds of vegetative growth and geo-climatic origins were also established for *M. anisopliae* var. *acridum* (Ouedraogo et al., 1997) and to a lesser extent for *B. bassiana* (Roberts and Campbell, 1977). In contrast, some studies reported no or weak correlation between the geoclimatic origin and the thermal tolerance of isolates in *M. anisopliae* (McCammon and Rath, 1994) and in *B. bassiana* (Fargues et al., 1997). The establishment of a strong relationship between the thermal tolerance of entomopathogenic isolates and their geoclimatic origin is hampered by the genetic complexity of the hyphomycetous species (Bridge et al., 1998) and the diversity of selection pressures, including the host origin and the geoclimatic constraints (Ferron et al., 1991). In spite of broad temperature ranges, these

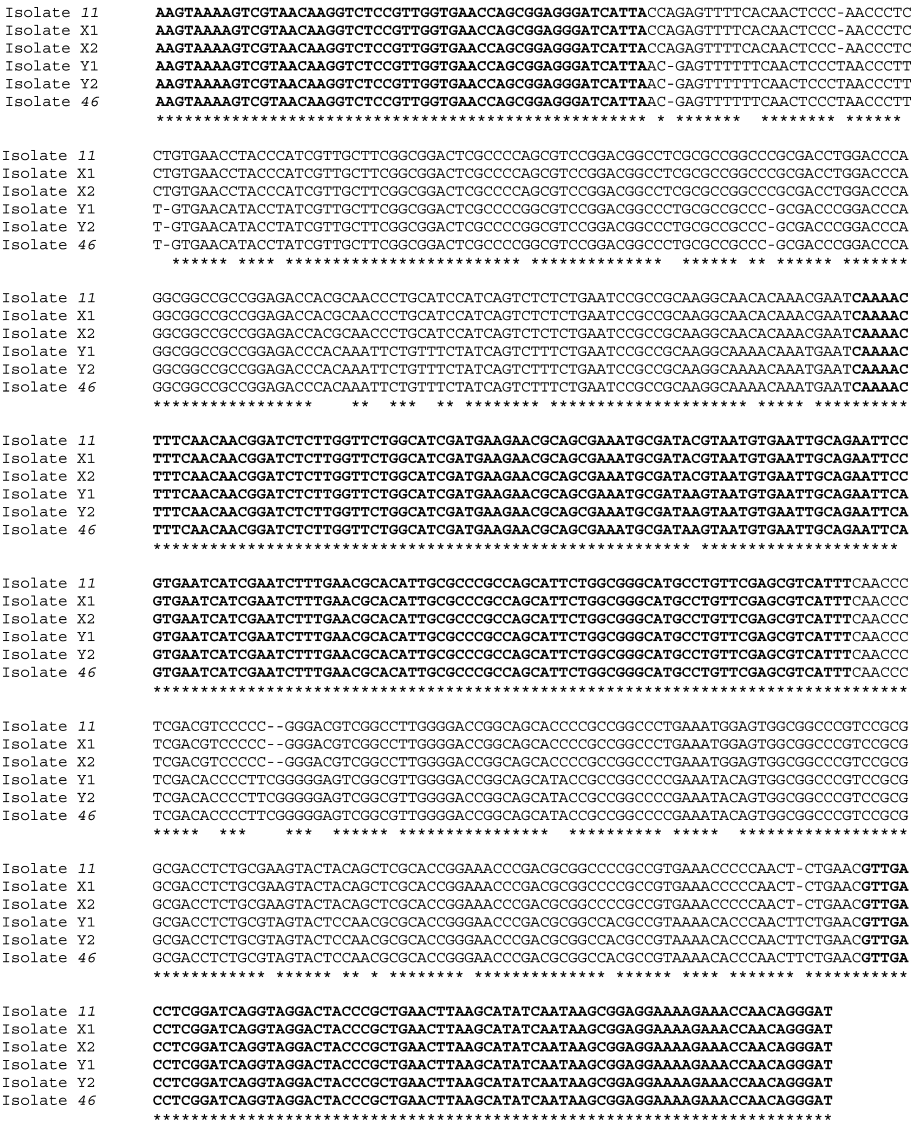

Fig. 5. Comparative sequence analysis of the ITS1-5.8S-ITS2 rDNA sequences from the six isolates *Pfr11* and *Pfr46*, *X1*, *X2*, *Y1* and *Y2*. The inferred positions of the 3′ end of the 16S rDNA, the 5.8S rDNA and the 5′ end of the 28S rDNA are in bold.

entomopathogenic hyphomycetous isolates exhibited differences in tolerance to extreme temperatures prevailing in the targeted environments (Inglis et al., 2001).

This study revealed that the two isolates *Pfr11* and *Pfr46*, which belong to different rDNA-ITS haplotypes (Fargues et al., 2002), also have different conidia dimensions. Recently, Sugimoto et al. (2003) established a correlation between conidial size and polymorphism length of the intergenic spacer (IGS) regions of ribosomal DNA (rDNA) in *L. lecanii* isolates. One distinct IGS haplotype grouping of six isolates produced larger conidia than the other isolates.

The study allowed a temperature profile to be formed for each isolate. In vitro growth of both isolates was adversely affected at low and intermediate temperatures ranging from 13 to 25 °C, where the isolate of temperate origin grew faster than the isolate from Pakistan. Inversely, at temperature regimes including high temperature at 35 °C, temperate isolate did not grow, while the isolate from Pakistan was the most active.

Under most temperature regimes, only one lineage was prevailing on the infected insect, whereas both lineages coexisted at 20–25 and 25-25 °C. Results of the second series of assays showed that, under these moderate temperature conditions, the relative frequencies of both lineage in in vivo produced conidia populations depended on the temperature preferences of each fungal isolate. Phenotypic analysis (conidial size) supported by molecular data (RFLP-ITS and RAPD) did not detect any genetic exchange between the two lineages in co-infected hosts under conditions given to be favorable to parasexual exchanges between vegetatively compatible fungal strains (Couteaudier and Viaud, 1997; Couteaudier et al., 1998).

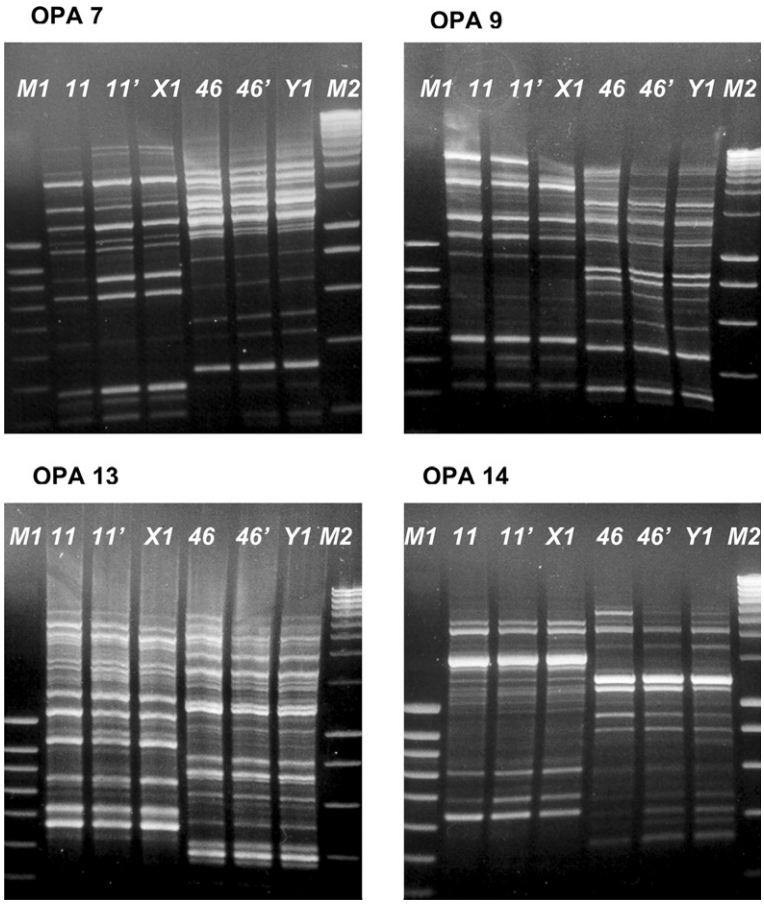

Fig. 6. RAPD-PCR patterns (generated by primers OPA7, OPA9, OPA 13, OPA14) of *P. fumosoroseus* reference strains host-passed (*11* and *46*) or not (*11′* and *46′*), and single-spore isolates (*X1* and *Y1*) from *G. mellonnella* co-infected with *Pfr11* and *Pfr46*. Eurogentec Smart Ladder SF of 1 kb (*M1*) and Smart Ladder of 10 kb (*M2*) were used as molecular-weight markers.

Our results demonstrated clearly that temperature acts as an important selective pressure upon the population complex which constitutes this entomopathogenic species. Such competition between incompatible VCGs which occurs under laboratory conditions, might also be observed under natural conditions. The use of formulated hyphomycetous propagules in novel environments tends to increase due to two main factors. First, the cosmopolitan nature of the major pest species contributes to the use of exotic fungal isolates in various environments (Fargues, 2003). However, this geographical extension favors competitive situations with local entomopathogenic populations that may be more adapted to the environmental constraints prevailing in the novel environments targeted for biocontrol approach. Second, the host ranges of the entomopathogenic isolates are not restricted to their original insect host species (Ferron et al., 1991; Vidal et al., 1997b), allowing enlargement of the market of each mycoinsecticide based on single fungal isolate formulation.

To date most of the fungal biocontrol agents have been selected based on their ability of pathogens to infect their target hosts, i.e., by means of virulence and host specificity bioassays (Butt and Goettel, 2000; Goettel and Inglis, 1997). However, co-infection studies have shown that temperature leads to the selection of the most environmentally adapted fungal genotypes. Bidochka (2001) and Bidochka et al. (2002) reported that insect host specificity is not the only predominant factor influencing the population genetics. Ecological fitness, implicating both insect habitat and pathogen temperature preferences should be also considered for the development of myco-insecticides (Bidochka, 2001; Bidochka et al., 2002; Wang et al., 2002).

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References

Bidochka, M.J., 2001. Monitoring the fate of biocontrol fungi. In: Butt, T.M., Jackson, C.W., Magan, N. (Eds.), Fungi as Biocontrol Agents. Progress, Problems and Potential. CAB International, Oxon, pp. 193–218.

Bidochka, M.J., Menzies, F.V., Kamp, A.M., 2002. Genetic groups of the insect-pathogenic fungus *Beauveria bassiana* are associated with habitat and thermal growth preferences. Arch. Microbiol. 178, 531–537.

Bolckmans, K., Sterk, G., Eyal, J., Sels, B., Stepman, W., 1995. PreFeRal, (*Paecilomyces fumosoroseus* (Wize) Brown and Smith, strain Apopka 97), a new microbial insecticide for the biological control of whiteflies in greenhouses. Med. Fac. Landbouww. Univ. Gent 60, 719–724.

Bridge, P., Couteaudier, Y., Clarkson, J.C., 1998. Molecular Variability of Fungal Pathogens. CAB International, Oxon, UK, p. 319.

Butt, T.M., Goettel, M., 2000. Bioassays of entomogenous fungi. In: Navon, A., Ascher, K.R.S. (Eds.), Bioassays of Entomopathogenic Microbes and Nematodes. CAB International, Oxon, UK, pp. 141–195.

Couteaudier, Y., Viaud, M., 1997. New insights into population structure of *Beauveria bassiana* with regard to vegetative compatibility groups and telomeric restriction fragment length polymorphisms. FEMS Microbiol. Ecol. 22, 175–182.

Couteaudier, Y., Viaud, M., Neuvéglise, C., 1998. Combination of different independent molecular markers to understand the genetic structure of *Beauveria* populations. In: Bridge, P., Couteaudier, Y., Clarkson, J. (Eds.), Molecular Variability of Fungal Pathogens. CAB International, Oxon, UK, pp. 95–104.

Fargues, J., 1976. Spécificité des champignons pathogènes imparfaits (Hyphomycètes) pour les larves de Coléoptères (Scarabaeidae et Chrysomelidae). Entomophaga 21, 313–323.

Fargues, J., 2003. New challenges for fungal bioinsecticides. IOBC/WPRS Bull. 26, 9–20.

Fargues, J., Luz, C., 1998. Effects of fluctuating moisture and temperature regimes on sporulation of *Beauveria bassiana* on cadavers of *Rhodnius prolixus*. Biocontrol Sci. Technol. 8, 323–334.

Fargues, J., Goettel, M.S., Smits, N., Ouedraogo, A., Rougier, M., 1997. Effect of temperature on vegetative growth of *Beauveria bassiana* isolates from different origins. Mycologia 89, 383–392.

Fargues, J., Bon, M.-C., Manguin, S., Couteaudier, Y., 2002. Genetic variability among *Paecilomyces fumosoroseus* isolates from various geographical and host insect origins based on the rDNA-ITS regions. Mycol. Res. 106, 1066–1074.

Fargues, J., Maniania, N.K., Delmas, J.C., Smits, N., 1992. Influence de la température sur la croissance in vitro d'hyphomycètes entomopathogènes. Agronomie 12, 557–564.

Ferron, P., Fargues, J., Riba, G., 1991. Fungi as microbial insecticides against pests. In: Arora, D.K., Ajello, L., Mukerji, L.G. (Eds.), Handbook of Applied Mycology, vol. 2. Dekker, New York, pp. 665–706.

Gilchrist, J.E., Campbell, J.E., Donnely, C.B., Peeler, J.T., Delanay, J.M., 1973. Spiral plate method for bacterial determination. Appl. Microbiol. 43, 149–157.

Goettel, M., Inglis, D., 1997. Fungi: Hyphomycetes. In: Lacey, L. (Ed.), Manual of Techniques in Insect Pathology. Academic Press, San Diego, CA, pp. 213–249.

Horsfall, J.G., 1956. Principles of Fungicidal Action. Chronica Botanica, Waltham.

Ignoffo, C.M., Garcia, C., Kroha, M.J., 1982. Susceptibility of larvae of *Trichoplusia ni* and *Anticarsia gemmatalis* to intrahemocoelic injections of conidia and blastospores of *Nomuraea rileyi*. J. Invertebr. Pathol. 39, 198–202.

Inglis, G.D., Goettel, M.S., Butt, T.M., Strasser, H., 2001. Use of hyphomycetous fungi for managing insect pests. In: Butt, T.M., Jackson, N., Magan, N. (Eds.), Fungi as Biocontrol Agents. Progress, Problems and Potential. CAB International, Oxon, UK, pp. 23–70.

Lacey, L.A., Fransen, J.J., Carruthers, R.I., 1996. Global distribution of naturally occurring fungi of *Bemisia*, their biologies and use as biological control agents. In: Gerling, D., Mayer, R. (Eds.), *Bemisia* 1995: Taxonomy, Biology, Damage, and Management. Intercept, Andover, UK, pp. 401–433.

Larena, L., Salazar, O., Gonzales, V., Julian, M.C., Rubio, V., 1999. Design of a primer for ribosomal DNA internal transcribed spacer with enhanced specificity for Ascomycetes. J. Biotechnol. 75, 187–194.

Luz, C., Fargues, J., 1998. Factors affecting conidial production of *Beauveria bassiana* from fungus-killed cadavers of *Rhodnius prolixus*. J. Invertebr. Pathol. 72, 97–103.

Masuda, T., Kikuchi, O., 1992. Pathogenicity of *Verticillium lecanii* isolates to whitefly and aphids. Jpn. J. Appl. Entomol. Zool. 36, 239–245, [in Japanese].

McCammon, S.A., Rath, A.C., 1994. Separation of *Metarhizium anisopliae* strains by temperature dependent germination rates. Mycol. Res. 98, 1253–1257.

Mietkiewski, R., Tzaczuk, C., Zurek, M., van der Geest, L.P.S., 1994. Temperature requirements of four entomopathogenic fungi. Acta Mycol. 29, 109–120.

Milner, R.J., Lozano, L.B., Driver, F., Hunter, D., 2003. A comparative study of two Mexican isolates with an Australian of *Metarhizium anisopliae* var. *acridum*—strain characterization, temperature profile and virulence for wingless grasshopper, *Phaulacridum vittatum*. Biocontrol 48, 335–348.

Osborne, L.S., Landa, Z., 1992. Utilization of entomogenous fungus *Paecilomyces fumosoroseus* against sweetpotato whitefly, *Bemisia tabaci*. IOBC/WPRS Bull. 17, 201–206.

Ouedraogo, A., Fargues, J., Goettel, M.S., Lomer, C.J., 1997. Effect of temperature on vegetative growth among isolates of *Metarhizium anisopliae* and *M. flavoviridae*. Mycopathologia 137, 37–43.

Roberts, D.W., Campbell, A.A., 1977. Stability of entomopathogenic fungi. In: Ignoffo, C.M., Hostetter, D.L. (Eds.), Environmental Stability of Microbial Insecticides. Entomol. Soc. Am., College Park, MD, pp. 19–76.

SPSS, Inc., 1997. SIGMASTAT User's Guide, version 2.03, SPSS Inc., Chicago. pp.

Sugimoto, M., Koike, M., Hiyama, N., Nagao, H., 2003. Genetic, morphological, and virulence characterization of the entomopathogenic fungus *Verticillium lecanii*. J. Invertebr. Pathol. 82, 176–187.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 24, 4876–4882.

Vey, A., Fargues, J., Robert, P.H., 1982. Histological and ultrastructural studies of factors determining the specificity of pathotypes of the fungus *Metarhizium anisopliae* (Metsch.) Sorok. for scarabeid larvae. Entomophaga 27, 387–397.

Vidal, C., Fargues, J., Lacey, L.A., 1997a. Intraspecific variability of *Paecilomyces fumosoroseus*: effect of temperature on vegetative growth. J. Invertebr. Pathol. 70, 18–26.

Vidal, C., Lacey, L., Fargues, J., 1997b. Pathogenicity of *Paecilomyces fumosoroseus*: (Deuteromycotina: Hyphomycetes) against *Bemisia argentifolii* (Homoptera: Aleyrodidae) with a description of a bioassay method. J. Econ. Entomol. 90, 765–772.

Wang, C.-S., Li, Z.-Z., Butt, T.M., 2002. Molecular studies of co-formulated strains of the entomopathogenic fungus, *Beauveria bassiana*. J. Invertebr. Pathol. 80, 29–34.

Zimmermann, G., 1986. The *Galleria* bait method for detection of entomopathogenic fungi in soil. J. Appl. Entomol. 102, 213–215.